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Basic-helix-loop-helix (bHLH) transcription factor proteins are involved in cell growth and differentiation, and the bHLH protein c-Myc has been implicated in breast cancer. We have determined that the sequence preference and specificity with which particular bHLH protein subclasses bind DNA are determined by binding conformations, which in turn are dictated by certain bHLH DNA binding region amino acids that are critical for protein function, but not for binding affinity. The data identify subtle effects on DNA binding that have important biological consequences, and reveal mechanisms by which different protein sub-family members can bind to different sites that share the CANNTG consensus.

The zinc finger protein TTP is induced by many growth factors, and is required for regulation of TNFα expression. We have shown that continuous expression of TTP or the related proteins TIS11b and TIS11d induces apoptosis and affects growth regulatory pathways. Like c-Myc, TTP also sensitizes cells to apoptosis induced by TNFα. Our data suggest that TTP is important in growth factor signaling, and that its role in TNFα function is likely to be complex. We have determined that these proteins interact with 14-3-3 proteins, which are involved in multiple signaling pathways, and are screening for additional TTP/TIS11 co-factors.

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Thomas Keith Blackwell 1/5/99

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INTRODUCTION:

Foreword:

As described in the previous report, and in accordance with the revised aims of this project, have concentrated our efforts on understanding basic-helix-loop-helix protein DNA binding specificity, and on elucidating the functions of TIS11/TTP proteins. Each section below will be divided into two parts, each of which will correspond to one of these projects.

Determinants of bHLH protein DNA binding specificity:

A large family of transcriptional regulators is defined by the basic-helix-loop-helix (bHLH) motif (Murre et al. 1989a), in which a DNA-binding basic region (BR) lies immediately amino terminal to the HLH dimerization segment (Davis et al. 1990; Murre et al. 1989b; Voronova and Baltimore 1990). bHLH proteins appear to be present in all eukaryotes and are involved in multiple different regulatory processes, including specification of a variety of different cell types (Weintraub et al. 1991a). Some bHLH protein family members appear to function as homodimers, but others may act together with a heterodimeric partner, with which they bind DNA at higher affinity (Weintraub et al. 1991a). For example, the closely-related bHLH proteins that mediate myogenic differentiation, including MyoD, appear to function as heterodimers with the widely-expressed 'E' group of bHLH proteins (Chakraborty et al. 1991; Davis et al. 1990; Lassar et al. 1991; Neuhold and Wold 1993). bHLH protein dimers generally bind to DNA at sites that contain the palindromic consensus CANNTG (Lassar et al. 1989), with each respective BR binding to a half site (Blackwell and Weintraub 1990; Ellenberger 1994; Ferre-D' Amare et al. 1993). Given the wide range of regulatory processes in which bHLH proteins are involved, and the apparent simplicity of the CANNTG binding consensus, it is an important question how different bHLH proteins act only on appropriate target genes.

In part, the specificity with which bHLH proteins function derives from preferential recognition of different classes of CANNTG sites by different bHLH protein subgroups. The HLH segment consists of a parallel, left-handed, four helix bundle, from which the BR extends N-terminally from helix 1 as an α-helix that crosses the major groove (Fig. 1) (Ellenberger et al. 1994; Ferre-D' Amare et al. 1993; Ma et al. 1994a). Crystallographic analyses have shown that the configuration of the HLH domain fold is remarkably preserved among bHLH proteins, but have revealed some differences in DNA recognition (Ellenberger et al. 1994; Ferre-D' Amare et al. 1993; Ma et al. 1994a). For example, Myc-family and other related bHLH proteins contain an arginine (Arg) residue at position 13 (Table 1), and bind preferentially to CACGTG or CATGTG sites (Blackwell et al. 1993; Dang et al. 1992; Halazonetis and Kandil 1992; Van Antwerp et al. 1992) because this Arg directly contacts bases in the center of the site (Ellenberger et al. 1994; Ferre-D' Amare et al. 1993). This sequence specificity distinguishes Myc-related bHLH proteins functionally from bHLH classes which have a different amino acid at BR position 13, and thus prefer different bases in the center of the CANNTG site (Blackwell and Weintraub 1990; Dang et al. 1992). However, structural analyses have not established a basis for the latter internal sequence preferences, or for the various examples of flanking sequence preferences

which have been described (Aksan and Goding 1998; Blackwell and Weintraub 1990).

Many bHLH proteins that do not have an Arg at position 13, including MyoD and other E protein partners (Fig. 1), bind to similar overlapping sets of DNA sequences but act on distinct sets of tissue-specific genes. Interactions with other DNA binding proteins at regulatory regions can contribute to these functional differences (Weintraub et al. 1994), but this specificity is not likely to derive entirely from other proteins that might be expressed only in particular cell lineages, because MyoD can induce multiple different cell types to differentiate into muscle (Weintraub et al. 1989). Consistent with the idea that the relationship between DNA-binding and transcriptional regulation by MyoD is complex, mutagenesis analyses have identified three "myogenic" residues within the BR and BR-HLH junction (Positions 5, 6, and 15; Fig. 1) that are not essential for DNA binding, but are required for induction of myogenesis (Davis et al. 1990; Davis and Weintraub 1992; Weintraub et al. 1991b). Corresponding residues appear to be required similarly for activity of Myogenin, a closely related myogenic bHLH protein (Brennan et al. 1991; Schwarz et al. 1992). These findings have suggested that, in myogenic bHLH proteins, the BR might be involved in protein-protein interactions when bound to DNA (Brennan et al. 1991; Davis et al. 1990; Weintraub et al. 1991b), and it has also been reported that these residues can be involved in interactions with other myogenic factors off DNA (Molkentin et al. 1995). In the MyoD crystal structure, residue 15 is oriented away from the DNA, but positions 5 and 6 are oriented toward the DNA (Ma et al. 1994a), indicating that any influence the latter residues might have on interactions with other proteins must be indirect, and mediated through the conformation of the DNA-bound BR. This mechanism could potentially contribute to target specificity, if only a subset of MyoD binding sites or promoter contexts were to allow binding in a conformation which would permit these protein-protein interactions to occur (Weintraub et al. 1991b ; Weintraub et al. 1994).

In this study, we have investigated how the myogenic BR residues and the BR/HLH junction influence DNA binding. We have determined that although these residues are not essential for binding of MyoD to a cognate site, they are critical for the overall sequence preferences and specificity with which it binds DNA. Particular mutations that abolish myogenic activity result in MyoD binding to DNA with the sequence preferences that are characteristic of the bHLH protein Twist, which *in vivo* is required for mesoderm formation (Michelson 1996), but may inhibit MyoD function (Huang et al. 1996; Spicer et al. 1996). These MyoD chimeras, Twist homodimers, and Twist/E2A complexes all bind preferentially to a CCATATGG consensus site, but with notably less specificity than wild-type MyoD binds to its preferred recognition sequence. Mutations of individual residues, or mispairing of the BR and BR-HLH junction, can alter preferences over the entire binding site, indicating that these residues influence the overall conformation of the protein-DNA complex. The results suggest that these classes of bHLH proteins recognize DNA through binding in one of a finite number of different conformations, each of which is associated with a particular set of binding sequence preferences. They also predict that the specificity of any co-factors that might recognize the bound BR would be determined by these conformations, and are consistent with the idea that subtle differences in binding sequence recognition

could be critical for restricting bHLH protein activity. The general principle that binding conformation establishes bHLH protein flanking sequence preferences is likely to be relevant to other subgroups of bHLH proteins, including the Myc-related group.

TTP/TIS11 proteins, cell proliferation, and TNFα activity:

TTP/TIS11/Nup475 (referred to as TTP, an abbreviation of Tristetraprolin) is an immediate-early gene which encodes a zinc finger protein that is induced transiently by serum treatment of cultured mammalian cells (DuBois et al. 1990; Lai et al. 1990; Varnum et al. 1991). Among the stimuli that induce TTP, or the related genes TIS11b and TIS11d, are insulin, TGFα, PDGF, FGF, TPA, LPS, TNFα, antibody-capping of B lymphocytes, and epidermal growth factor (EGF), which has been implicated in breast cancer (Bustin et al. 1994; Carballo et al. 1998; Corps and Brown 1995; Gomperts et al. 1992; Mittelstadt and DeFranco 1993; Wang et al. 1994). The TTP gene is also induced *in vivo* during regeneration of certain types of tissue, such as liver and intestine (DuBois et al. 1990; Ehrenfried et al. 1995)

Of the three mammalian TTP/TIS11 genes, only TTP has been disrupted in mice. The TTP-/- mice have a syndrome of myeloid hyperplasia, polyarthritis, autoimmunity, cachexia, alopecia, and, dermatitis, a constellation of symptoms which is very similar to that resulting from transgenic expression of tumor necrosis factor-α (TNFα) (Taylor et al. 1996a). This syndrome is prevented by regular injections of anti-TNFα antibodies (Taylor et al. 1996a), and it depends upon presence of the TNFα receptors, indicating that it is mediated by TNFα (Carballo et al. 1997). Transplantation experiments indicate this syndrome is caused by non-lymphoid hematopoietic cells, and although systemic levels of TNFα are normal in these mice, their macrophages can over-produce TNFα in response to LPS *in vitro*, suggesting an abnormality in TNFα expression (Carballo et al. 1997). In addition, the TNFα mRNA appears to be relatively more stable in TTP -/- cells, and over-expressed TTP protein can both destabilize various mRNAs that have similar 3' sequences which regulate their relative stabilities, and be cross-linked to the TNFα mRNA (Carballo et al. 1998). These experiments suggest the model that TTP acts directly to regulate TNFα mRNA stability (Carballo et al. 1998). This model is both exciting and provocative, but considerable molecular analyses remain to be done to establish it as the cause of the phenotype observed in TTP -/- mice. In addition, it is possible that this phenotype is also influenced by indirect effects of a lack of TTP, such as an effect on signaling pathways involved in lymphoid cell proliferation, or in auto-feedback regulation of TNFα expression (see below). An understanding of how TTP/TIS11 proteins function at the molecular level will not only illuminate a genetically-identified key step in TNFα action, but will also provide insights into molecular regulatory circuits that are involved in the responses to multiple different growth factors, including EGF.

TTP/TIS11 proteins are defined by two tandem zinc fingers of the Cys-Cys-Cys-His (CCCH) class (Fig. 4) (Worthington et al. 1996), a type about which relatively little is known (Berg and Shi 1996). Humans and rodents have three TTP/TIS11-related genes (TTP, TIS11b and TIS11d), which are very similar within these zinc fingers (Fig. 4; not shown) and in size, but are otherwise divergent. TTP and TIS11b/d orthologs have also been identified in Drosophila, Xenopus, C. elegans,

and yeast (De et al. 1999; Ma and Herschman 1995; Ma et al. 1994b)(Fig. 4). Genetic and molecular studies have implicated other proteins that contain CCCH zinc fingers in mRNA processing, or in gene regulation at the transcriptional or post-transcriptional levels (Barabino et al. 1997; Guedes and Priess 1997; Mello et al. 1996; Seydoux et al. 1996; Zhang 1992). For example, the C. elegans PIE-1 protein (Fig. 4) appears to encode a broadly-acting repressor of mRNA transcription that is essential for establishment of the germline (Batchelder et al. 1999; Mello et al. 1996; Seydoux and Dunn 1997; Seydoux et al. 1996), and also appears to have post-transcriptional functions (C. Mello and G. Seydoux, pers. comm.). However, the role played by its CCCH zinc fingers is unknown. Some CCCH zinc finger proteins bind RNA, or are associated with RNA-binding proteins (Bai and Tolias 1996; Barabino et al. 1997; Murray et al. 1997; Zhang 1992). However, despite this association with RNA binding, it has not been determined at the molecular level exactly how any CCCH zinc fingers function.

In cultured mammalian fibroblasts, TTP is detected in the nucleus (DuBois et al. 1990), but it translocates to the cytoplasm when its expression is up-regulated during the earliest phases of growth factor responses (Carballo et al. 1998; Taylor et al. 1996b). TIS11b and d may be expressed more constitutively, but are also induced by multiple different growth factors (Corps and Brown 1995; Gomperts et al. 1992). In adult mice, TTP is expressed in organs that contain lymphoid tissue (Taylor et al. 1996a), but relatively little is known about the embryonic pattern or cell-type specificity of TTP/TIS11 gene expression. In both Drosophila (Ma et al. 1994b) and Xenopus (De et al. 1999), these genes are expressed in the earliest embryonic stages, suggesting that they have broad and perhaps overlapping functions. In the fission yeast S. pombe, lack of a TTP/TIS11 ortholog prevents pheromone-induced sporulation and mating (Kanoh et al. 1995). This mutation can be partially overcome by constitutive expression of the appropriate MAP kinase, or an activated *ras* gene, but over-expression of the TTP/TIS11 gene cannot overcome defects at various points in the signaling cascade (Kanoh et al. 1995). These observations are important, because they indicate that the TTP/TIS11 protein is required for this signaling response to function or to be effected, but is not an integral component of the pathway (Kanoh et al. 1995). In S. cerevisiae, overexpression of a TIS11b/d-related protein can complement simultaneous temperature-sensitive mutations in the cell-cycle regulators *nim-1* and *cdc25*, suggesting that it can promote proliferation (Warbrick and Glover 1994). On the other hand, in a different study, overexpression of TTP (or of related yeast genes) appeared to retard growth of S. cerevisiae (Thompson et al. 1996). This complex picture is consistent with TTP/TIS11 proteins regulating expression of genes involved in responses to various diverse growth factors, perhaps including regulation of genes involved in cell cycle entry.

We have determined that forced expression of TTP in mammalian cell lines induces apoptotic cell death and affects cell cycle progression. This finding appears to be analogous to the observation that expression of c-Myc (Evan et al. 1992) or CDC25 (Galaktionov et al. 1996), which induce proliferation, can cause apoptosis if growth factors are not present. Lack of growth factors enhances induction of apoptosis by TTP, but is not required for this effect. Like c-Myc, TTP also acts synergistically with TNFα to induce apoptosis. The results suggest that TTP can

promote cell-cycle entry or affect cell-cycle progression, consistent with its putative involvement in growth factor responses, and that its constitutive expression results in a regulatory perturbation that triggers programmed cell death. We have also determined that TIS11b and TIS11d have analogous effects, except that they are less effective at synergizing with TNFα to induce apoptosis, a finding that could be relevant to the phenotype of the TTP -/- mice. We have also begun efforts to identify factors that interact with these proteins, and have obtained preliminary findings that they bind to 14-3-3 proteins.

BODY:

Determinants of bHLH protein DNA binding specificity:

When the MyoD BR is replaced with that of E12 (an E2A protein), the resulting mutant protein (MD(E12B); Fig. 1) binds to a muscle-specific regulatory element as a heterodimer with E2A, but will not induce myogenesis or activate transcription through a complex muscle-specific enhancer (Davis et al. 1990; Davis and Weintraub 1992; Weintraub et al. 1991b). Similar results have been obtained when the E12 BR was substituted into the myogenic bHLH protein Myogenin (Brennan et al. 1991). Back-substitution of A_5 of MyoD into MD(E12B) does not enhance its activity, but substitution of both A_5 and T_6 restores the capability of either MyoD or Myogenin to induce myogenesis in cell culture assays (Fig. 1)(Brennan et al. 1991; Weintraub et al. 1991b)

To test whether these mutations might have subtle effects on DNA binding that were not detected previously, we have used the selection and amplification of binding sites (SAAB) technique of *in vitro* nucleic acid selection (Blackwell and Weintraub 1990) to identify sequences that are preferentially bound by these proteins. Using sequence libraries in which positions within and flanking the CANNTG bHLH consensus were randomized, we have performed multiple reiterative rounds of selection for binding. Since the CANNTG consensus fixes the position of binding, the resulting selected sites can be sequenced as a pool (Blackwell et al. 1990; Blackwell and Weintraub 1990), making this strategy a very sensitive indicator of binding preferences that might not be identified through conventional approaches.

As shown in the previous report, in this assay, MyoD binds preferentially to the sequence G/AACAGCTGTT/C ((Blackwell and Weintraub 1990); not shown), but MD(E12B) prefers the sequence G/ACCATATGGT/C, which differs from the MyoD site over the 8 central base pairs (not shown). This site also differs significantly from the CACCTG sequence preferred by E2A proteins (Blackwell and Weintraub 1990), indicating that the binding preference of the E12 BR can vary depending upon its molecular context. Back-substitution of A_5 of MyoD into MD(E12B) results in preferences that are more MyoD-like at positions ±4, but otherwise has little effect on binding (MD(E12B-A); Fig. 1; not shown). However, substitution of both A_5 and T_6, which restores the capability to induce myogenesis in cell culture assays (Brennan et al. 1991; Weintraub et al. 1991b), results in preferences that are indistinguishable from those of wild-type MyoD (MD(E12B-AT); Fig. 1; not shown). The data indicate that substitution of these individual amino acid residues influences binding preferences over the entire range of each 5 bp half-site.

To investigate how heterodimer formation influences DNA binding by these proteins, we have performed SAAB experiments on various MyoD and E12 mutant combinations. These experiments were also described in the previous report. In general, the basic region dictates half-site preferences in these respective heterodimer combinations. However, surprisingly, heterodimers of the MD(E12B) and E12(MDB) mutants (Fig. 1), prefer sites that are more similar to those selected by MD(E12B), including the AT sequence in the center of the site (not shown). This last observation indicates that binding preferences are affected over the entire site when both BRs are mis-paired relative to their protein contexts, and suggests that BR positioning is important for determining binding sequence preferences.

The binding sites prefered by MD(E12B) and MD(E12B-A) homodimers, and by MD(E12B) + E12(MDB) heterodimers, are remarkably similar to the preferences of the bHLH protein Twist (Fig. 1). As described previously, Twist homodimers and Twist+E12 heterodimers both prefer the core sequence CATATG (not shown), which corresponds to natural Twist-responsive regulatory elements (Szymanski and Levine 1995). They also select MyoD-like sequences at ± 5, and are similar to MD(E12B-A) in their preferences at ± 4 (not shown). The data are consistent with the notion that the A_5, N_6 sequence common to Twist and MD(E12B-A) BRs (Fig. 1) is involved in mediating the corresponding preferences observed at ± 4 and ± 5. However, it is surprising that the Twist core preferences are identical to those associated with mis-pairing of both MyoD and E12 BRs, because these last proteins lack obvious BR similarities aside from the conserved residues (Fig. 1). Together, these observations suggest that the common element dictating all of these preferences is the conformation of the BR when it is bound to DNA, and that this conformation is influenced by residues at the BR-HLH junction. A surprising aspect of these experiments is that the "Twist-like" binding preference which is characteristic of MD(E12B) and Twist complexes is generally less specific than the preferences of MyoD and other bHLH proteins. This conclusion was determined through a series of binding site competition analyses that were described in the previous report, and it supports the idea that this preference derives from a common binding conformation shared by these proteins.

An interesting aspect of our experiments is that the myogenic activity of the MD(E12B) derivatives correlates with their DNA binding preferences, as described previously. Mutagenesis experiments have also indicated that the BR-HLH junction region is critical for myogenesis. An E12 protein that contains the MyoD basic region (E12/MDbasic) is incapable of inducing myogenesis (Fig. 1), but introduction of only four additional MyoD residues confers myogenic activity (E12/MDBJ; Fig. 1). Remarkably, an E12 protein containing only the three "myogenic" MyoD residues, A_5 and T_6 from the BR, and K_{15} from the junction region (E12/AT,K; Figure 3), can transactivate a muscle-specific reporter and induce myogenesis in tissue culture assays (Fig. 1). Because we have observed that the junction region can affect binding conformation, we have begun to address the role of the individual junction residues in establishing binding sequence preferences.

We have constructed a series of mutants in which non-essential MyoD residues have been substituted with alanine (Fisher et al. 1993), and residues at BR/junction positions 5, 6, and 15 have been substituted with the corresponding amino acids that are present in the E2A and Twist proteins (Fig. 2). These mutants

will allow us to investigate the DNA-binding effects of these BR and junction residues in a more defined context. Introduction of N6 into an alanine-substituted BR (MD-AAATA, Fig. 2) confers preference for a Twist-like site, indicating that this residue alone is sufficient (Figs. 3A and 3B, lanes 8 and 9). In contrast, substitution of junction residues alone does not appear to influence binding preference significantly (Figs. 3A and 3B, lanes 3-7). It will now be important to test whether particular BR/junction combinations establish the binding preferences of the respective bHLH proteins from which they were derived, and to test these conclusions in binding competition and titration experiments.

TTP/TIS11 proteins, cell proliferation, and TNFα activity:

The apparent role of TIS11/TTP proteins in growth factor/mitogen/cytokine responses suggests that expression of these proteins might influence cell cycle entry or progression, and that analysis of this effect could identify regulatory circuits through which they normally function. As discussed in the previous report, forced expression of TTP from a CMV-based vector (CS2) induces NIH3T3 cells to undergo cell death through apoptosis, as indicated by the morphology of TTP-transfected cells (Fig. 5). In these apoptosis assays, TTP-transfected cells can be identified by co-transfected β-galactosidase (β-gal) or green fluorescent protein (GFP) markers (Fig. 5). Considerable apoptosis is apparent at 24 hrs. after transfection, and by 48 hours post-transfection the majority of TTP-expressing cells are dead or in the process of dying (Fig. 6A). Providing further support for the notion that this cell death is apoptotic, a TUNEL assay detects presence of double-stranded DNA breaks in TTP-transfected cells (not shown), and this cell death can be inhibited by co-transfection of the Bcl-2 protein (not shown).

Similar results have been obtained using various human cell lines, including HeLa cells and the osteogenic sarcoma cell line U2OS (Fig. 6B-6C). TTP also induces apoptosis in SAOS2 cells (Fig. 6D), an osteogenic sarcoma cell line that lacks both the retinoblastoma (Rb) and p53 proteins, indicating that they are not required for this apoptotic effect. In this last cell line, the decreased extent of apoptosis at 24 hr. may be linked to its longer division time (not shown). These experiments indicate that TTP expression affects regulatory pathways that are critical for cell growth and survival.

TTP and the TIS11b and TIS11d proteins are similar in size and related in amino acid sequence throughout their entire lengths, particularly in their zinc finger regions (Fig. 4), suggesting that they might have similar functions. Supporting this idea, when transfected into NIH3T3 cells, each of these three proteins induces apoptosis at a similar frequency and rate (Fig. 7), suggesting that they act on common or overlapping intracellular targets.

Like TTP, the c-Myc protein is expressed in the initial stages of growth factor responses (Luscher and Eisenman 1990). Although c-Myc expression is associated with cell proliferation, it induces apoptosis in cells that have been deprived of growth factors (Evan et al. 1992). This apoptosis can be inhibited by PI-3 kinase/Akt signaling that emanates from growth factor receptors (Franke et al. 1997; Franke et al. 1995), suggesting that it derives occurrence of an intracellular proliferative signal (from c-Myc) in the absence of an extracellular growth stimulus. The TIS11/TTP proteins induce apoptosis in the presence of growth factors (Fig. 7), suggesting that

they may trigger additional apoptotic or proliferative pathways. However, in addition, the extent to which these proteins induce apoptosis is significantly augmented by serum deprivation and associated growth arrest (compare 10% and 0.1 serum, Fig. 8). This last finding indicates that growth factor signaling ameliorates the apoptotic effects of these proteins, supporting the idea that this apoptosis involves an effect on signaling pathways that are linked to cell cycle entry or progression.

Expression of c-Myc also induces apoptosis synergistically with the cytokine TNFα, in an effect that appears to involve G1 cyclins (Janicke et al. 1996). In most cell types, TNFα induces apoptosis only if protein synthesis is inhibited by cycloheximide, because it also induces genes that block the apoptotic signal (Beg and Baltimore 1996; Liu et al. 1996; Van Antwerp et al. 1996; Wang et al. 1996). The synergy between TNFα and c-Myc could involve an effect on these protective genes, but the apparent link to G1 cyclins suggests that an effect on the cell cycle is likely to be involved, a model that is supported by the observation that TNFα-stimulated apoptosis is blocked by inhibition of cyclin-dependent kinases (Meikrantz and Schlegel 1996).

We have determined that forced TTP expression also synergizes with TNFα treatment to induce programmed cell death. At 24 hr. post-transfection, even low levels of TNFα triggers considerable apoptosis in TTP-expressing cells in the absence of cycloheximide (Fig. 9). In contrast, even though TIS11b and TIS11d induce apoptosis with a frequency similar to that of TTP, they do not act synergistically with TNFα (Fig. 9). Our findings support the idea that TTP triggers apoptosis through growth regulatory pathways that overlap with those stimulated by c-Myc. In addition, they reveal a functional distinction between TTP and TIS11b and TIS11d, a finding that could be relevant to the mis-regulation of TNFα observed in TTP -/- mice.

To investigate directly how TIS11/TTP proteins function in the cell, we have begun efforts to identify protein factors with which they interact. We have performed a two-hybrid screen between TTP and a mixed-stage mouse embryo cDNA library, using a low-copy system (Vidal 1997) because TTP expression is toxic to the yeast cell (not shown). From this screen we have isolated a number of proteins that interact with TTP. Most of these interactors are still being characterized, but we have begun to study one of them, the 14-3-3 eta (η) protein (Fig. 10). In the two hybrid assay, 14-3-3η binds to TTP and TIS11b, but not to TIS11d or to various control baits (Fig. 10). Further supporting the idea that these interactions are specific, TTP and TIS11d both contain sequences that are predicted to be 14-3-3 recognition sites (not shown) (Yaffe et al. 1997) (M. Yaffe, pers. comm.). When TTP that has been expressed in transfected cells is tested for binding to other GST-linked 14-3-3 isoforms, with the exception of 14-3-3ε each of the other six 14-3-3 proteins also binds to TTP, but some of these isoforms bind to different phosphorylated forms of TTP (not shown). 14-3-3 proteins are found in all eukaryotes, and they bind primarily to phosphorylated serine residues (Yaffe et al. 1997) but also to some unphosphorylated sites (Petosa et al. 1998). They have been implicated in multiple regulatory pathways, including ras/MAP kinase signaling (Chang and Rubin 1997; Roberts et al. 1997; Thorson et al. 1998; Tzivion et al. 1998; Xing et al. 1997), G2/M cell cycle arrest in both humans and yeast (Forbes et al. 1998;

Hermeking et al. 1997; Peng et al. 1997), and control of apoptosis (Yano et al. 1998; Zha et al. 1996). Each of these regulatory pathways is potentially relevant to TTP function.

CONCLUSIONS:

Our studies of bHLH protein DNA binding preferences have shown that the "myogenic" residues in the MyoD BR, A_5 and T_6, have a marked effect on DNA binding preference, which is also influenced by residues in the BR-HLH junction. They suggest that these bHLH proteins can bind DNA in particular conformational "states" that are determined by residues 5, 6, and the junction, and that these "states" establish preferences for different CANNTG sites. Our current mutagenesis experiments will investigate more directly the effects of these two BR residues and of individual junction residues on DNA binding affnity and specificity. These experiments are underway and will be completed in February, 1999. A manuscript will then be prepared that describes these experiments, along with those described in previous progress reports.

Our studies of TIS11/TTP proteins have demonstrated that their continuous expression induces cultured cells to undergo apoptosis, demonstrating that TTP can have broader effects on cellular regulatory pathways than is suggested directly from the analysis of TTP -/- mice. Our findings that serum deprivation sensitizes cells to this effect, and that TTP sensitizes cells to apoptosis stimulated by TNFα, support the model TTP affects cell growth regulatory pathways. Also supporting this idea, continuous TTP expression decreases the fraction of cells in the G1 phase, and increases the G2/M fraction (not shown). Of the three TIS11/TTP proteins, only TTP sensitizes cells to TNFα, an observation that is consistent with the idea that lack of TTP could also influence the response of cell to TNFα. Experiments are underway to test whether TTP-induced apoptosis occurs at the G2/M boundary, and whether increases in cyclin-dependent kinase or MAP kinase activity are involved.

By performing a two-hybrid screen and initiating biochemical efforts to identify TTP-interacting factors, we have begun to open up new avenues for investigating how TTP functions at the molecular level. In addition to screening for other proteins that interact with TTP, we will investigate how 14-3-3 protein expression might be involved in TTP-stimulated apoptosis by over expressing wild-type and dominant negative 14-3-3 forms (Thorson et al. 1998), and by identifying and mutating the 14-3-3 binding site(s) in TTP. In a collaboration we have recently initiated with members of the Brown lab at Stanford, we will also use microarray technology (Iyer et al. 1999) to search for additional potential mRNA targets of TTP. Such experiments will be important for gaining a mechanistic understanding of how TTP is involved in TNFα activity, and of how the three TTP/TIS11 proteins are involved in responses to various growth factors.

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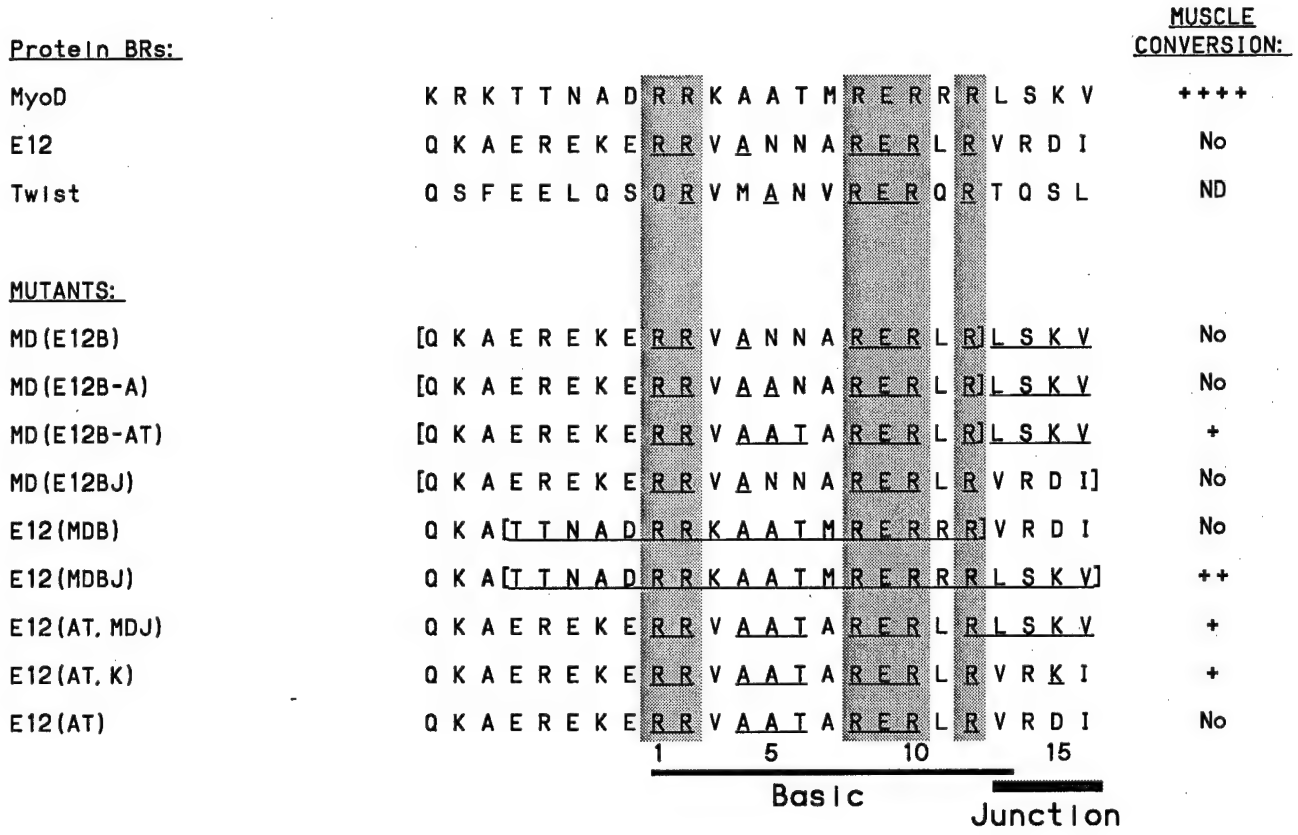

Figure 1. Basic region and junction mutants and their myogenic properties. These mutants in the MyoD and E12 proteins have been described previously (Weintraub, et al., 1991; Davis and Weintraub, 1992). Each of them will bind to a muscle-specific regulatory sequence *in vitro*. Muscle conversion refers to their capability of inducing transfected cultured fibroblasts to differentiate into muscle. The "++" represents 30-50% of the myogenic conversion frequency obtained with wild-type MyoD, and the "+" represents 5-30% of the wild-type frequency. Residues that are identical to MyoD are underlined, and substituted BR and junction regions are bracketed.

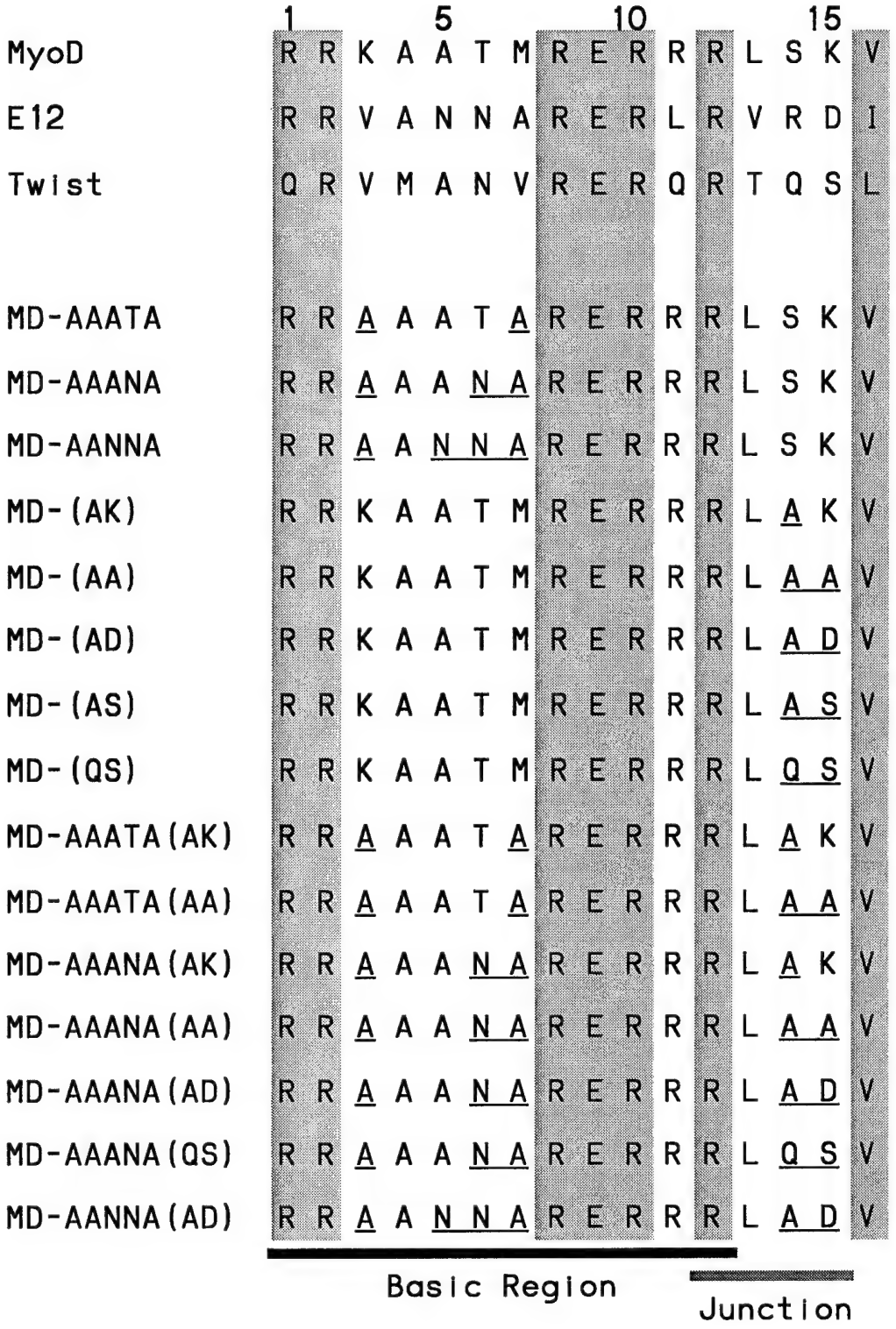

Figure 2. MyoD basic region mutants. These mutations have been introduced into full-length MyoD by the Quick-change method (Stratagene) or by PCR. Substituted positions have been underlined.

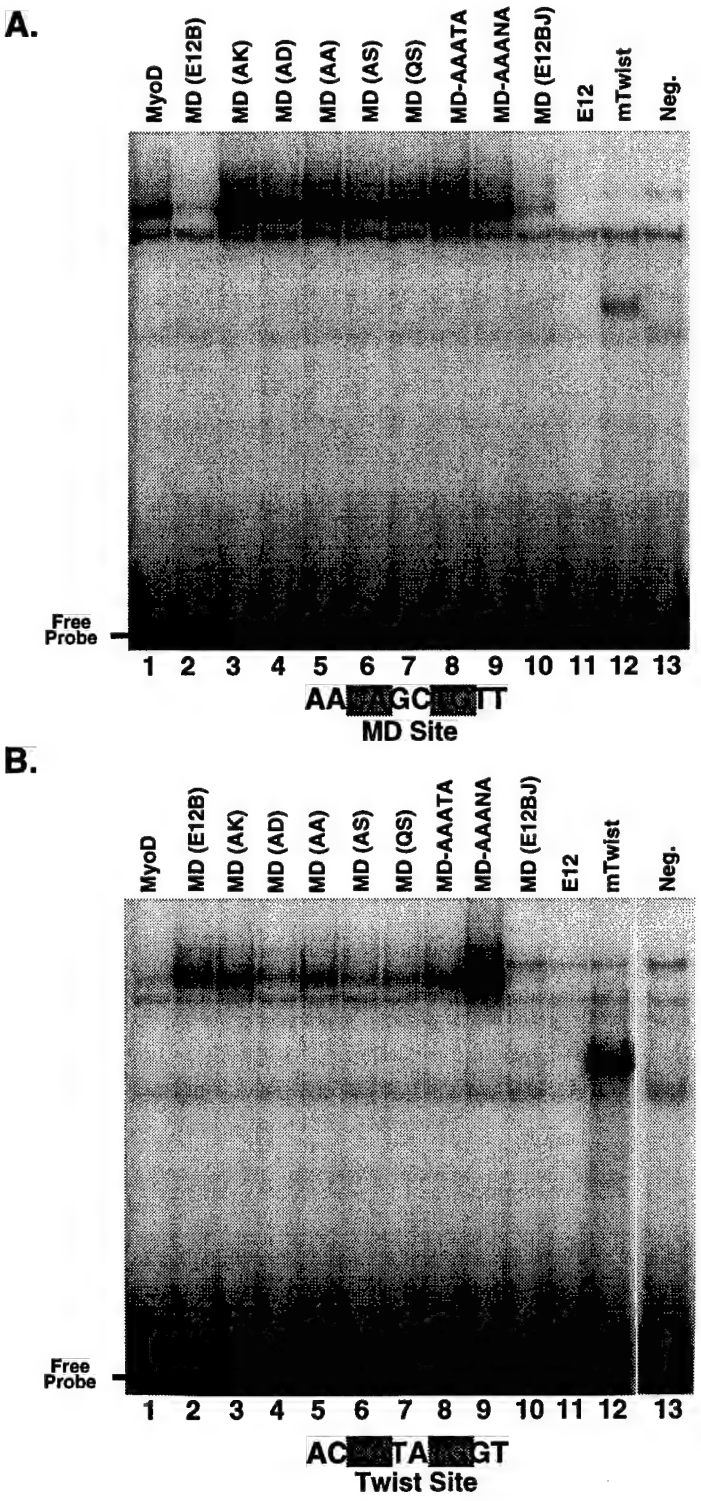

Figure 3. A preliminary electrophoretic mobility shift assay of DNA binding by the indicated MyoD mutants, which are described in Fig. 2. These proteins were expressed by *in vitro* translation, then analyzed for binding to the indicated sites in the presence of poly dI:dC competitor. The DNA sites were identical except for the sequences shown. Approximately equal amounts of each protein was assayed, except for MyoD, which was present at a slightly lower concentration.

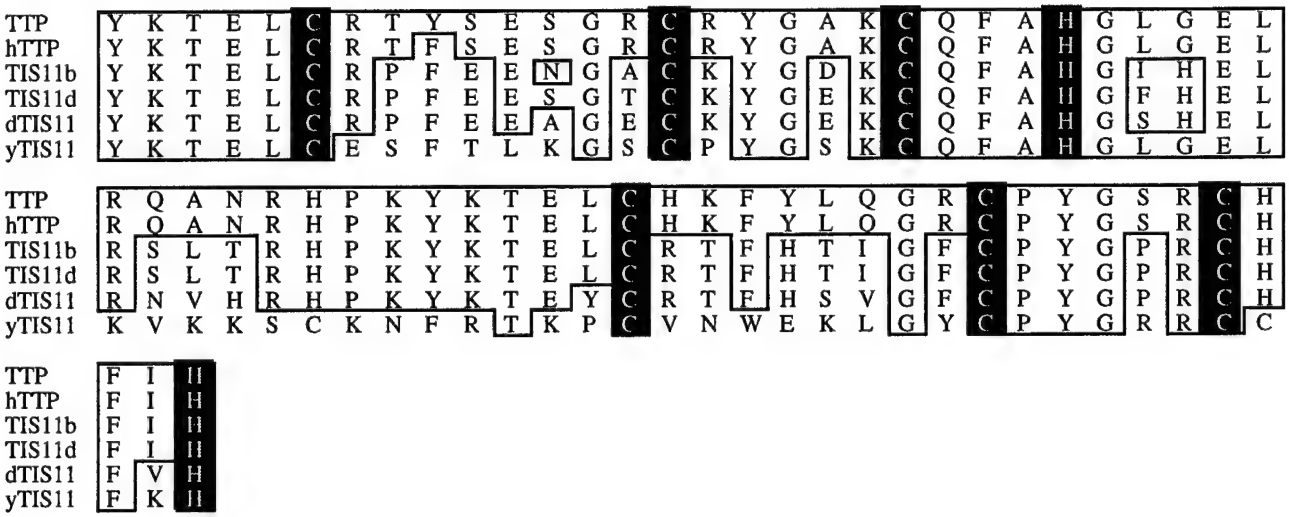

Figure 4. Alignment of the zinc finger regions from TTP family members. Human sequences are represented by h, Drosophila by d, and yeast (S. cerevisiae) by y. Residues that are identical to TTP are boxed, and amino acids that are predicted to contact zinc (Worthington, et al., 1996) are shaded.

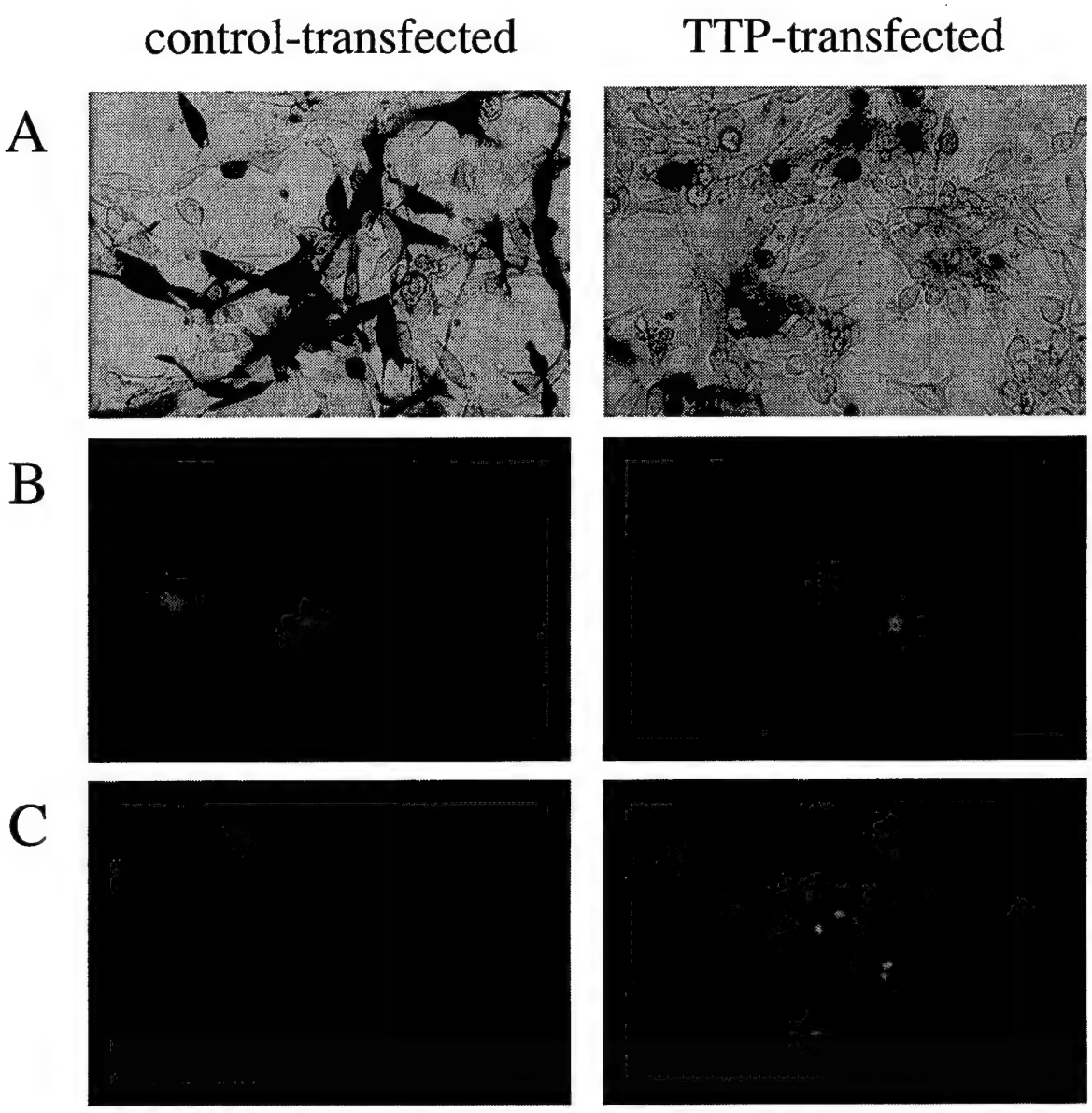

Figure 5. Cell death in NIH3T3 cells caused by forced expression of TTP. Cells were transiently transfected with either an empty expression vector (CS2; left hand side) or a TTP expression plasmid (CS2TTP; right hand side). (A) Cells were transfected by lipofection with 1.5μg of effector plasmid plus 0.5 μg of a β-galactosidase reporter plasmid, then stained for reporter expression after 48 hours. Note that in the TTP-transfected field, cells which stain blue have the shriveled appearance characteristic of apoptotic cells. (B) and (C) show a similar experiment in which TTP was introduced into these cells together with a green fluorescent protein (GFP) expression plasmid. GFP expression identifies transfected in (B), and (C) (an identical field to B) shows by Hoechst staining the presence of pycnotic nuclei in the TTP-transfected fluorescent cells.

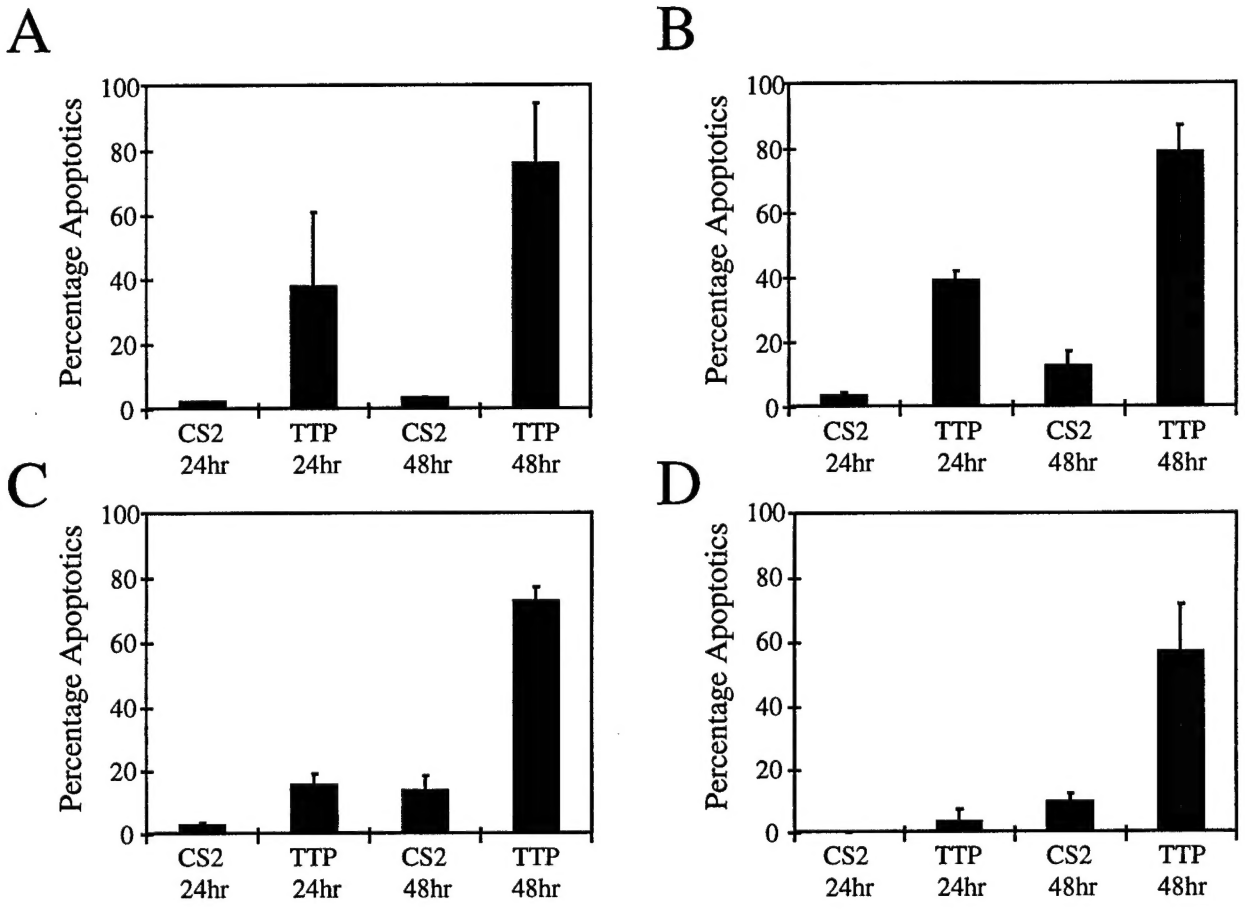

Figure 6. Overexpression of TTP causes death in NIH3T3 (A), HeLa (B), U2OS (C), and SAOS2 (D) cell lines. In each experiment, 200ng of TTP or CS2 empty vector control were co-transfected along with 100ng of β-galactosidase reporter plasmid and 1.8 μg of pBluescript DNA. Cells were stained for β-galactosidase activity 24 or 48 hours later, and percentage dead blue cells scored. Results show representative experiments in which each bar represents the mean of 3 (A) or 4 (B-D) transfections, and error bars represent standard deviations.

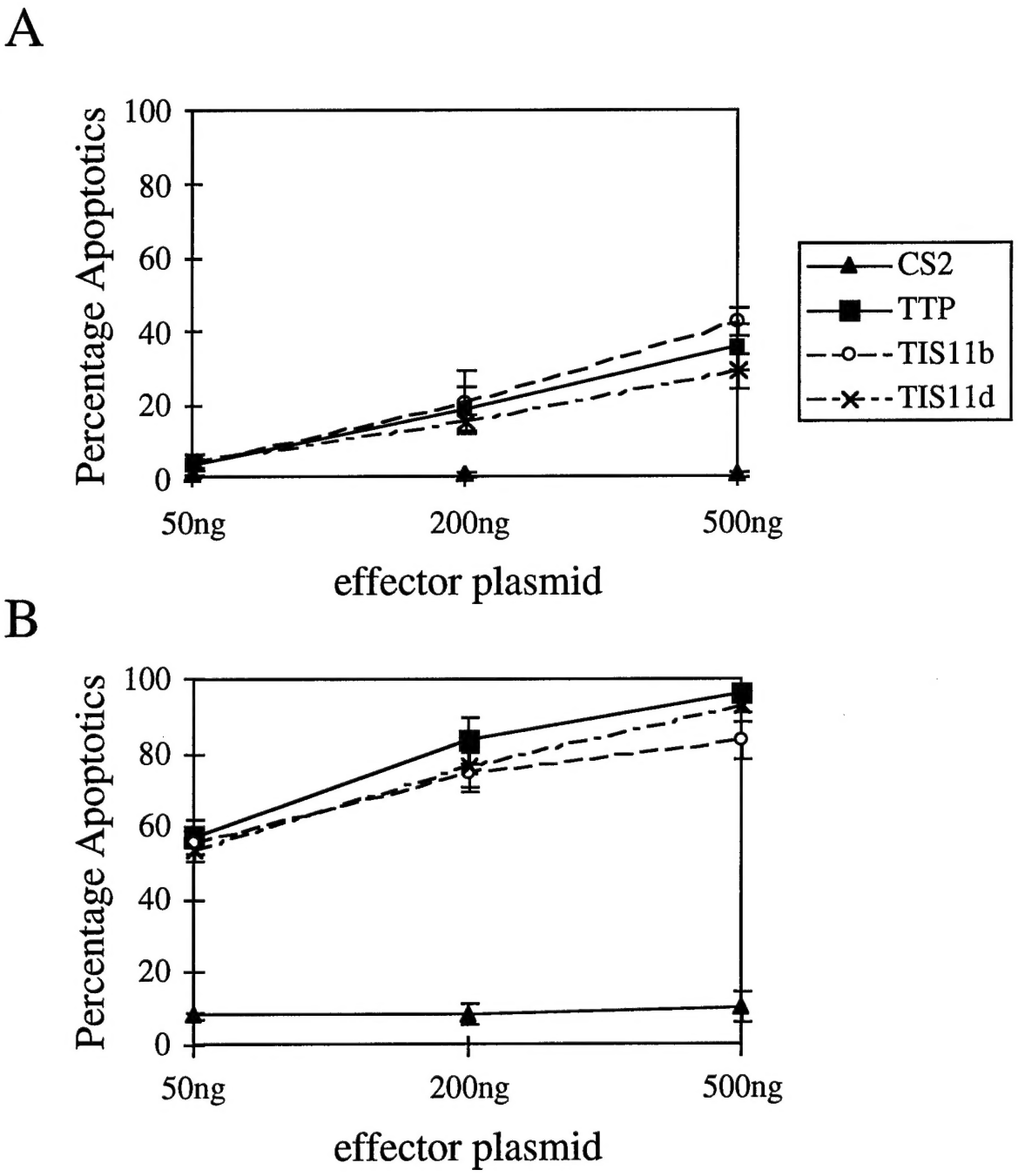

Figure 7. Apoptosis induced by TTP, TIS11b and TIS11d expression. The indicated amounts of the indicated CS2 effector plasmids were co-transfected into NIH3T3 cells with 100ng of β-galactosidase reporter plasmid, and pBluescript to equalise the total transfected DNA to 2μg. Cells were stained and the percentage of dead blue cells counted at either 24 hours (A), or 48 hours (B) aftertransfection. Graphs show a representative experiment in which each data point represents the mean of 4 transfections, and error bars show standard deviations.

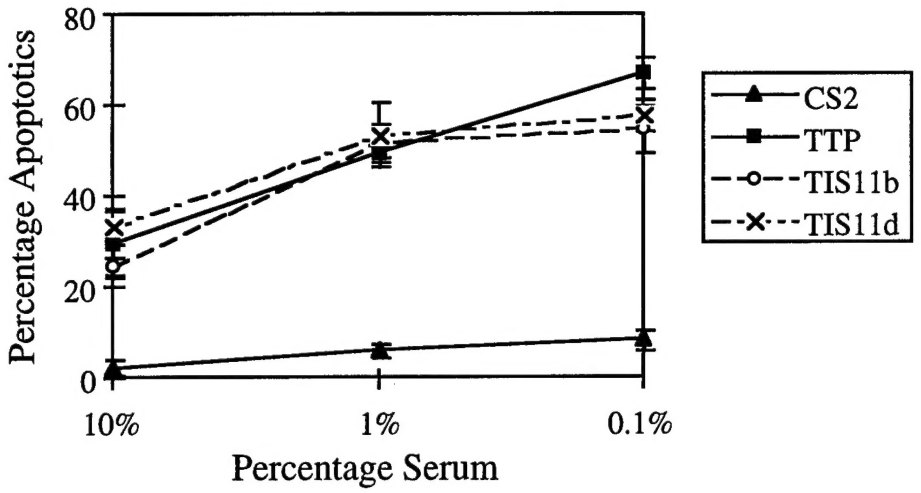

Figure 8. Serum deprivation enhances apoptosis induced by TTP, TIS11b and TIS11d. Transfections were carried out as described in Fig. 7, but incubated in medium containing the indicated amounts of serum. After 24 hours, cells were stained for β-galactosidase activity, and the percentage of dead blue cells counted.

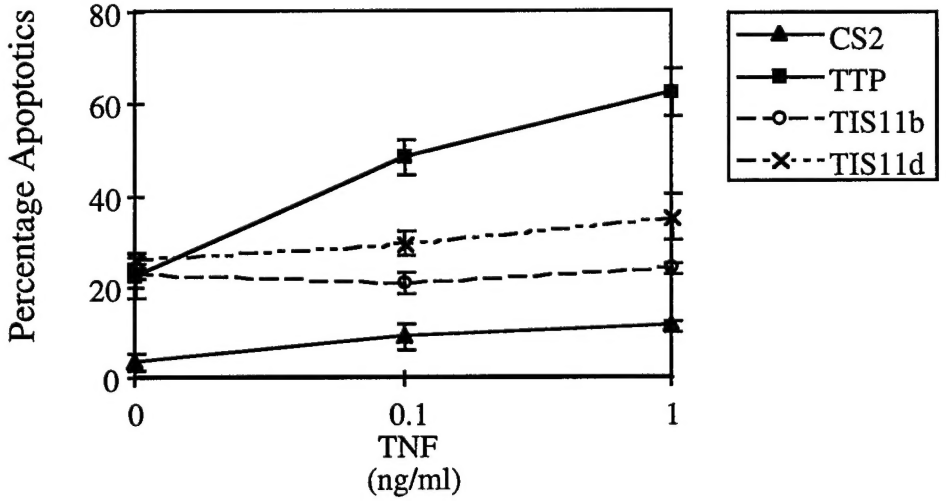

Figure 9. Synergistic induction of apoptosis by TTP/TIS11proteins and TNFα. 50ng of effector plasmid, 100ng of β-galactosidase expression plasmid and 1.85 μg of pBluescript were transfected into NIH3T3 cells, which were then treated with the indicated concentrations of TNFα. After 24 hours, cells were stained, and the percentage of dead blue cells counted.

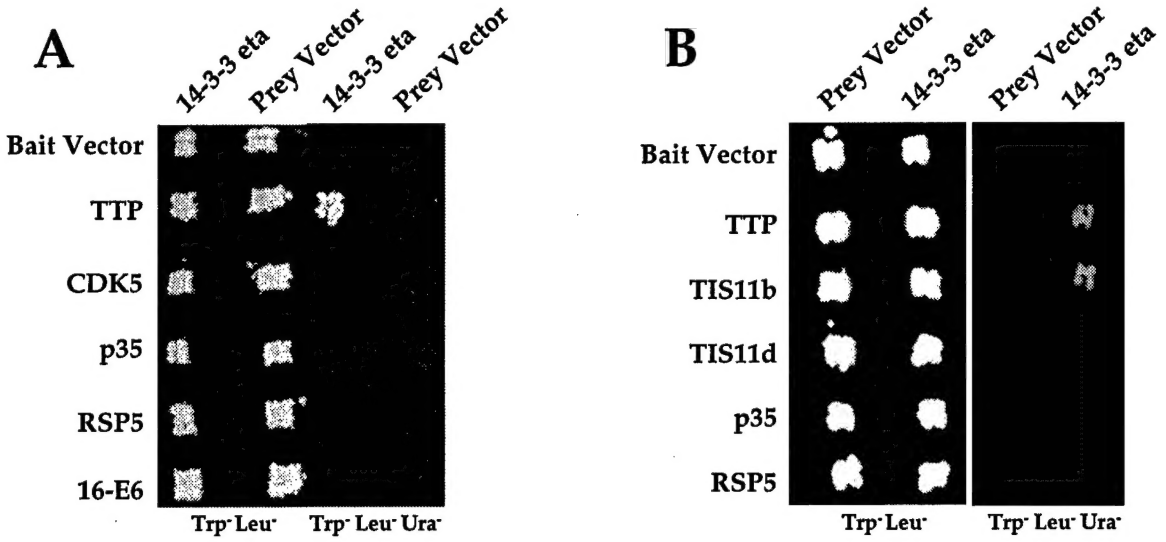

Figure 10. Interaction between TTP/TIS11 proteins and 14-3-3η. (A) A cDNA clone encoding 14-3-3η was isolated from a mouse embryonal cDNA library by two-hybrid screening with full-length mouse TTP. This mating assay shows that this 14-3-3η fusion protein interacts with a TTP bait, but not with a variety of other proteins, as indicated by growth on Ura- medium. (B) A mating assay which indicates that this 14-3-3η protein interacts with TIS11b, but not TIS11d.